IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

N. KIMURA ET AL. : GROUP ART UNIT: 1644

SERIAL NO.: 09/963,316 :

FILED: SEPTEMBER 25, 2001 : EXAMINER: VANDERVEGT, FRANCOIS P

FOR: USES OF ANTI-CX3CR1 ANTIBODY, ANTI-FRACTALKINE ANTIBODY AND FRACTALKINE

DECLARATION UNDER 37 C.F.R. 1.132
ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231
SIR;

I, Toshio Imai, a citizen of Japan, one of the inventors of the above-identified application, hereby declare and state that:

The following experiments were conducted by me or under my direct supervision.

Human FKN was produced as a fusion protein (FKN-SEAP) with secreted alkaline phosphatase (SEAP)-(histidine)$_6$. By using binding of FKN-SEAP to CX3CR1 receptor expressed on the B300.19 cell surface as an index, the binding inhibitory activity of anti-FKN monoclonal antibodies was examined.

(1) Preparation of fusion protein (FKN-SEAP)

To express FKN as a fusion protein with SEAP, the ORF of FKN cDNA was inserted between of *Sal*I and *Xba*I sites of an expression vector pDREF-SEAP(His)$_6$ to prepare a vector pDREF-FKN-SEAP(HIS)$_6$ encoding a protein in which FKN is fused with SEAP-(HIS)$_6$ through a linker composed of five amino acids (Ser-Arg-Ser-Ser-Gly) (Imai et al., Cell. 1997 Nov 14;91(4):521-30). This vector was introduced into 293/EBNA-1 cells (Invitrogen) by using Lipofectamin (Gibco-BRL). After 3 or 4-day culture, culture supernatant was collected, passed through a filter with a pore size of 0.45 μm and stored at 4°C with addition of 20 mM HEPES (pH 7.4) and 0.02% sodium azide.

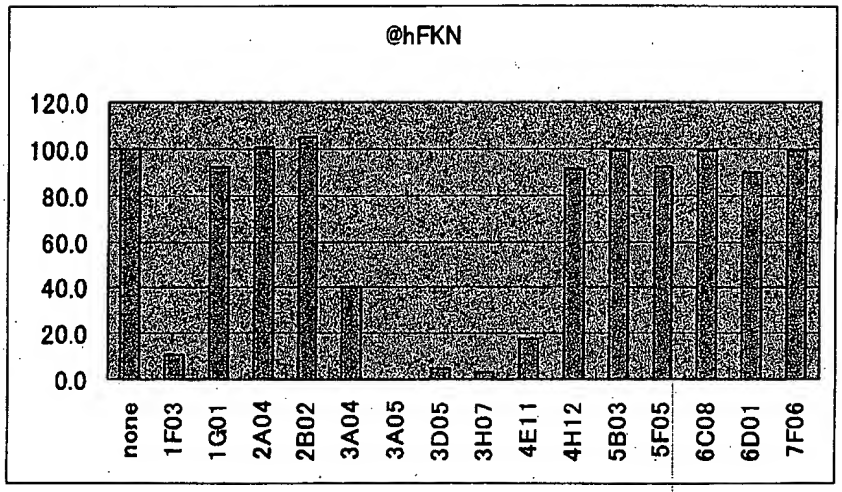
The concentration of FKN-SEAP in the culture supernatant is determined in terms of alkaline phosphatase (AP) activity. The alkaline phosphatase (AP) activity

was measured according to a chemiluminescence method by using Great EscApe Detection Kit (Clontech) as relative light unit (RLU)/s. An AP standard curve was prepared by using purified PLAP (Cosmo Bio).

(2) Effect of anti-FKN monoclonal antibody on binding of FKN-SEAP fusion protein to CX3CR1

Monoclonal antibody-producing hybridomas were prepared as described in Example 4 in the specification of the above-identified application. The used antigen was the extracellular domain of FKN as described in Imai T. et al., Cell 91:521-530, 1997. By ELISA, it was confirmed that all of tested monoclonal antibodies bound to the extracellular domain of FKN.

A change of an amount of FKN-SEAP binding to CX3CR1-expressing B300.19 cells when the culture supernatant of the anti-FKN monoclonal antibody-producing hybridoma is added was examined. Specifically, 50 µl of the culture supernatant of the hybridoma, 50 µl of 4 nM FKN-SEAP in a binding solution (RPMI-1640 containing 20 mM HEPES (pH 7.4), 1% BSA, and 0.02% sodium azide), and 100 µl of CX3CR1-expressing B300.19 cell suspension adjusted to 2 x 10^5 cell/100 µl with the binding solution were mixed to react them at 16°C for 1 hour. The cells were washed five times with 150 µl of the binding solution and lysed with 50 µl of 10 mM Tris-HCl (pH 8.0) containing 1% Triton X-100. The phosphatase derived from the cells was inactivated by a treatment at 65°C for 10 minutes. Then, the AP activity in the cell lysate was measured to determine the amount of FKN-SEAP which bound to CX3CR1. The binding activity was calculated by taking the binding amount in the absence of the anti-FKN antibody as 100%. The results are shown in the following graph. Among the tested anti-FKN antibodies, 6 clones showed the binding inhibitory activity, but 9 clones showed no inhibitory activity.



I hereby declare that all statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 3/24/2005

Toshio Imai

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